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The Impact of Infrared Synchrotron Radiation in Biology: Past, Present, and Future

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PAST

Unlike many x-ray based spectroscopies, which were made possible by the advent of synchrotron radiation, infrared (IR) spectroscopy has been used for many years without the benefits of a synchrotron source. Commercial IR spectrometers are equipped with conventional thermal (globar) sources that provide IR power that is comparable to the IR radiation emitted from a synchrotron. However, the primary advantage of synchrotron IR light is its brightness (defined as the photon flux or power emitted per source area and solid angle), which is 100-1000 times greater from a synchrotron source (Figure 1) [1]. This brightness advantage is not because the synchrotron produces more power, but because the effective source size is small and the light is emitted into a narrow range of angles.

High brightness is desirable for any measurement with a limited "throughput", meaning either a small sample area, the requirement for a narrow beam, or a combination of both. Microspectroscopy is perhaps the best-known example of a measurement with low throughput, and the synchrotron source is well suited to this technique.

IR microscopes equipped with conventional IR sources have been used to examine biological samples for nearly 20 years. IR microspectroscopy has been used to examine numerous plant and animal tissues [2]. For complex samples such as human tissues, an IR spectrum provides a direct indication of sample biochemistry. Figure 2 illustrates IR spectra of a common phospholipid (DMPC, dimyristoylphosphatidycholine), protein (hemoglobin), and nucleic acid (a polynucleotide). The dominant absorption features in the lipid spectrum are found in the region 2800-3000 cm^{-1}, and are assigned to asymmetric and symmetric stretching vibrations of CH_3 (2956 and 2874 cm^{-1}) and CH_2 (2922 and 2852 cm^{-1}) groups of the acyl chains. In addition, the strong band at 1736 cm^{-1} arises from ester C=O groups in the lipid. The protein spectrum has two primary features, the amide I (1600-1700 cm^{-1}) and amide II (1500-1560 cm^{-1}) bands, which arise from specific stretching and bending vibrations of the peptide backbone. The frequency of the amide I band is particularly sensitive to protein secondary structure. The nucleic acid spectrum also displays C=O stretching vibrations from the purine (1717 cm^{-1}) and pyrimidine (1666 cm^{-1}) bases. In addition, the region between 1000-1500 cm^{-1} contains contributions from asymmetric (1224 cm^{-1}) and symmetric (1087 cm^{-1}) PO_2^- stretching vibrations.

The assignments of various spectral features in biological samples have been the subject of numerous publications, which have been reviewed recently [3]. However, it should be emphasized that these frequencies are

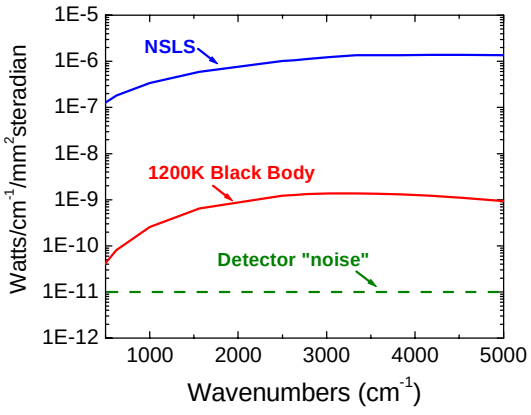

Figure 1. *Synchrotron radiation is 2-3 orders of magnitude brighter than conventional globar sources as shown in this plot of the power delivered to a 10 μm sample at f/1. An estimate of the signal to noise can also be seen.*

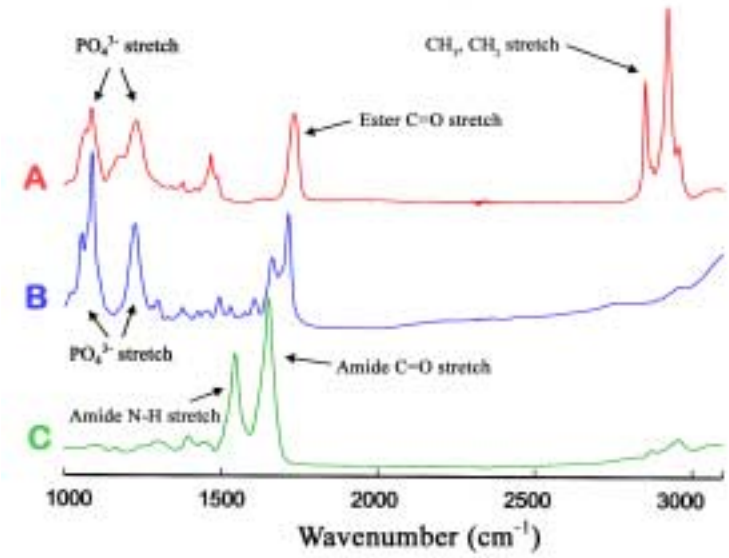

Figure 2. *Various cellular components have dramatically different IR spectra as demonstrated by IR spectra of (A) a lipid (dimyristoylphosphatidylcholine, DMPC), (B) a poly-nucleic acid, and (C) a protein (hemoglobin). For all spectra, films were prepared on BaF_2 disks and 128 scans were collected at 4 cm^{-1} resolution using a globar source.*

offered as guidelines only, and that the recipe for assignment of IR absorption features in biological samples requires knowledge of both sample histology and pathology, in addition to spectroscopy. Armed with this information, variations in nucleic acid, protein, and lipid content or structure, can provide important details about the chemistry of diseased states. For example, aggregates of misfolded proteins, i.e. amyloid plaques, have been identified in the brain tissue of Alzheimer's disease patients [4]. Spectral evidence of cervical cancer [5, 6], heart disease [7] and bone diseases such as osteoarthritis, osteoporosis, and osteogenesis imperfecta [8-10] have been identified. In addition, contaminants in human tissue, such as silicone in breast tissue [11] and narcotics in human hair [12, 13] have also been observed.

Although conventional IR microspectroscopy has proven extremely valuable for resolving the chemical components in biological samples, the long wavelengths of IR radiation limit the spatial resolution that can be achieved. When considering the available spatial resolution, two issues should be taken into account. The first consideration is the acceptable signal-to-noise ratio (S/N), which decreases as apertures are closed to confine the IR beam to smaller areas. The second issue is diffraction. Existing instruments using a conventional IR source encounter a S/N limitation when apertures confine the IR to an area of 20-30 μm in diameter. This constrains the analysis of biological specimens to the tissue level only. Individual biological cells are typically 5-30 μm in diameter, making them too small to probe with a conventional IR source.

The high brightness of the synchrotron source allows smaller regions to be probed with acceptable S/N [14, 15]. Indeed, aperture settings smaller than the wavelength of light can be used; though in this case, diffraction controls the available spatial resolution [16]. Thus for a typical biological specimen, the diffraction-limited spatial resolution for primary lipid (C-H stretch), protein (amide I), and nucleic acid (P-O stretch) absorption features is approximately 3, 6, and 12 μm, respectively. The improvement in spatial resolution achieved by using a synchrotron IR source has only been realized recently, and applications to biological systems are still in their infancy.

PRESENT

The high spatial resolution of a synchrotron IR source permits the chemical mapping of single living cells for the first time. Individual mouse hybridoma B cells have been examined during necrosis and also during the end phases of mitosis [17]. In these experiments, a cytospin was used to deposit cells onto BaF_2 disks. This technique does not kill the cells, but rather removes excess solution while keeping them hydrated, which suspends their activity for several hours. IR maps are collected during this time by automated scanning of the sample on a precision X,Y micro-stage. After >8 hours, the cells eventually dehydrate and die. In another study, similar cells were examined during the process of apoptosis (i.e. programmed cell death), where a single cell was probed in 50:50 D_2O/H_2O buffer for several hours while apoptosis progressed (J.L. Teillaud, *et al.*, to be published). Evidence of

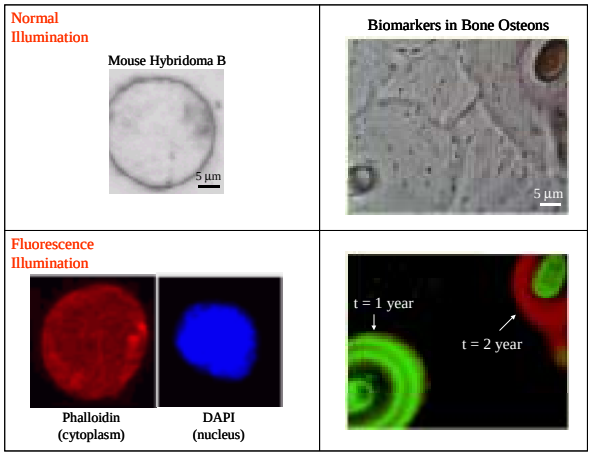

Figure 4. *Normal (top) and fluorescence (bottom) illumination of a mouse hybridoma B cell (left) and flourochrome labels in bone osteons (right). In the mouse cell, phalloidin is used to label the cytoplasm and DAPI is used to label the nucleus. In the bone tissue, fluorochromes deposited into newly deposited bone signify bone that is 1 year old (green) and two years old (orange).*

protein aggregation and degradation was clearly observed in the frequency of the amide I band.

With the ability to probe smaller and smaller areas with the synchrotron IR microscope, new techniques are currently being applied to aid in sample visualization. For example, 5 µm-wide layers of newly deposited bone have been simultaneously visualized with fluorescence microscopy and probed with the IR microscope [18, 19]. Other visualization techniques include the use of polarized light and differential interference contrast (DIC). On the cellular and sub-cellular level, these techniques can be used to visualize fluorescent tags bound to particular cellular components and even antibodies to individual proteins. Once identified, the IR microscope can be used to analyze the chemical environment in and around that region of interest. It should be noted that fluorescent labels are generally present in extremely low (i.e. nanomolar) concentrations, so they do not interfere with the IR technique; they are used exclusively for visualizing a region of interest. Figure 3 illustrates some examples of fluorescence visualization techniques that are particularly useful for examining biological specimens.

Since synchrotron radiation has opened the field of IR microspectroscopy to the level of cellular and sub-cellular chemical mapping, it is becoming increasingly evident that methods for accurate and objective data analysis and interpretation are needed. Spectral interpretation is often a highly subjective process, a fact that is made worse when one considers the many hundreds of spectra that are often acquired from a single cell or tissue area. These issues were first realized with the development of IR focal plane array detectors, which enable individual spectra to be collected at each pixel simultaneously, dramatically increasing the data acquisition rate [20, 21]. To date, these detectors have not been used with synchrotron sources.

To address these concerns, pattern recognition techniques are currently being developed and applied to IR data and correlated with biochemical or histological data. These methods help to remove subjectivity and allow realistic processing of large data sets. The mapping of specific functional group intensities and frequencies is perhaps the most straightforward method. Using a synchrotron IR source, functional group mapping has proven extremely informative in the analysis of single living cells [17], bone tissue [22], and Alz-

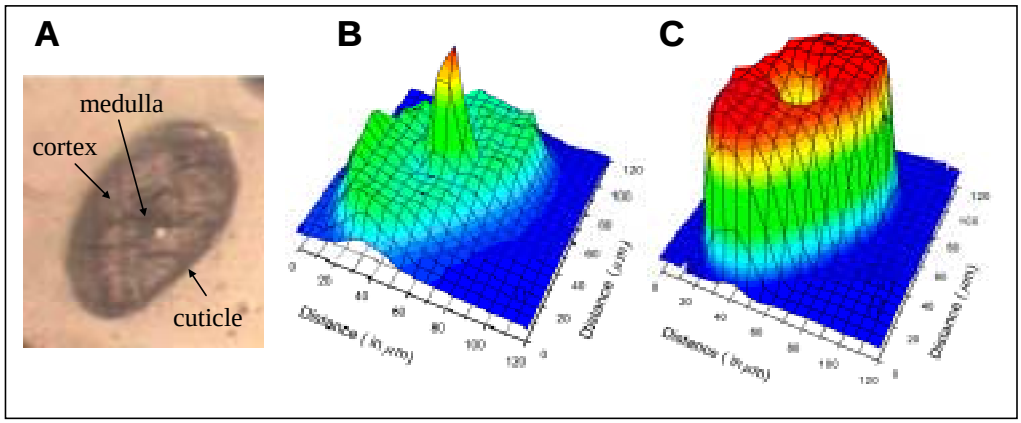

Figure 3. *(A) Optical image of a transverse-cut section of human hair illustrating the various regions: medulla, cortex, and cuticle. Functional group mapping of the hair can be seen for (B) the lipid distribution (2919 cm^{-1}) and (C) the protein distribution (1650 cm^{-1}). The lipid concentration is highest in the medulla, whereas the protein content is highest in the cortex. IR spectra were collected with a 3x3 µm aperture in 3 µm steps, 32 scans/point, and 4 cm^{-1} resolution.*

heimer's diseased brain [4]. Most recently, an investigation of human hair has demonstrated that the medulla (5-20 μm diameter), cortex (40-100 μm), and cuticle (<5 μm) regions of the hair can easily be resolved using a synchrotron source. Figure 4 illustrates the dramatic differences in the composition of these regions.

More sophisticated pattern recognition techniques, such as cluster analysis and linear discriminate analysis, utilize intrinsic features of the IR spectra. Perhaps the earliest application of pattern recognition techniques to biological samples involved the analysis of bacterial strains, which were identified by their IR spectra using this approach [23]. Other applications include the identification of cholesterol accumulation in the liver [24], the assignment of breast tumor grade [25], and the assessment of Alzheimer's disease severity in brain tissue [26].

One particular limitation of these types of spectral analyses is the requirement for proper assignment of "control" spectra to a particular class of tissue or cellular component. Using a conventional IR source, limited spatial resolution often results in a single spectrum containing an average of tissues and cell types present in the sample. The high spatial resolution of the synchrotron IR source dramatically improves the ability to obtain representative spectra from *pure* tissue and even cellular components.

In addition to data analysis, many new methods are being developed which allow presentation of these complex data sets in a form readily interpreted by the non-expert. Gray scale functional group mapping and digital staining have been demonstrated [27] and an example can be seen in Figure 5. In digital staining, 8-bit gray scale images showing the distribution of three tissue components are converted to 8-bit red, green, and blue scale images. These three 8-bit images are then combined to produce 24-bit color images. By superimposing the chemical information contained in the separate functional group maps, digital staining provides a means of visually representing relative proportions of chemical species.

FUTURE

The high brightness of the synchrotron IR source has opened the door to cellular and sub-cellular chemical imaging of living cells. With enhanced visualization techniques, sub-cellular components can be investigated. Moreover, the combination of high spatial resolution with excellent S/N will permit the examination of ongoing cellular processes in real-time. Statistical methods will play an important role in the analysis of large IR spectroscopic data sets, although this field is still in its infancy. Effective presentation of these data with methods such as gray scale imaging and digital staining will be important factors in determining the degree to which IR microspectroscopy becomes generally accepted as a tool for cellular and sub-cellular characterization.

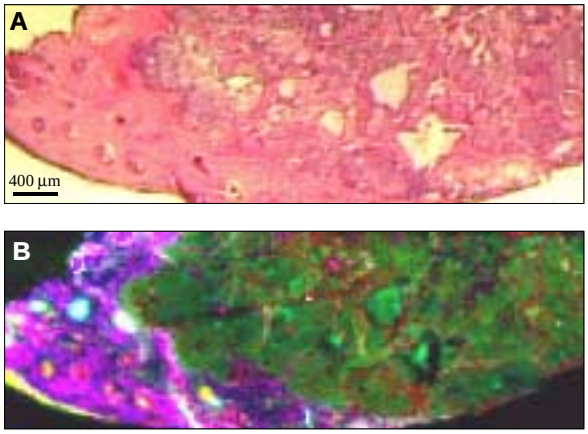

Figure 5. *(A) Optical image and (B) 24-bit color digital stain of a section of skin tumor. The digitally stained map is produced by combining color-scaled maps for collagen (blue), lipid (red) and nucleic acid (green). Results demonstrate that the dermis contains a high proportion of collagen with some lipid (blue/purple), the tumor is comprised mainly of nucleic acids (green), and the epidermis contains high proportions of both nucleic acids and lipids (yellow).*

In addition to microspectroscopic imaging, methods for studying protein folding and reaction dynamics on a sub-millisecond time scale are also being developed through the use of a unique rapid-mix IR flow cell [28]. Time-dependent phenomena can be probed on even shorter (i.e. sub-nanosecond) time scales by taking advantage of the pulsed nature of the synchrotron source [29]. No other pulsed IR source spans the large spectral range produced by a synchrotron. In addition, significant flux in the far-IR regime continues to prove useful for the analysis of low frequency, collective modes in proteins [30]. Microspectroscopy in the far-IR spectral range has also recently been demonstrated [31] and will likely be expanded upon in the future as well.

The pursuit of improved spatial resolution with the synchrotron IR source will continue into the future, based on both conventional (far-field) and possibly near-field techniques. For example, increasing the optical system numerical aperture by "immersing" the specimen in a high index material (e.g. KRS-5, ZnSe, germanium) can lead to factors of two or higher improvements in the spatial resolution. In a related technique, germanium planar waveguide technology has

been used to isolate single Xenopus laevis oocytes membranes and identify protein and lipid components [32]. Near field techniques, where a small (submicron) source of IR is scanned very near to the specimen, have been demonstrated with IR laser sources [33, 34], and similar technology may be applicable to synchrotron radiation microspectroscopy as well.

SYNCHROTRON INFRARED FACILITIES WORLDWIDE

Facilities for IR synchrotron radiation can be found throughout the world, serving to produce light for the scientific community. The National Synchrotron Light Source (NSLS, Brookhaven National Laboratory) presently operates six IR beamlines, making it a premier synchrotron facility for IR investigations [35]. Active IR beamlines can also be found at UVSOR, Okasaki (Japan); ALS, Berkeley and SRC, Stoughton (USA). In Europe, IR activities continue at the SRS, Daresbury (UK); LURE, Orsay (France); MAXLAB, Lund (Sweden); and at Daφne, Frascati (Italy). Other facilities that are either planning or considering IR programs include Diamond, Rutherford Lab (UK); BESSY II, Berlin, ANKA, Karlsruhe and DELTA, Dortmund (Germany); Duke-FEL, Durham (USA); CLS, Saskatoon (Canada); LNLS, Campinas, (Brazil); CAMD, Baton Rouge, (USA); SURF-3, Gaithersburg (USA); NSRL, Hefei (China); SPring8, Nishi-Harima (Japan); and SRRC, Hsinchu (Taiwan). Based on the increasing interest in synchrotron IR radiation, it is clear that the application of synchrotron IR spectroscopy to biological problems has a bright future.

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